Effects of lactic acid bacteria cultures on pathogenic microbiota from fishes

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Headline: Inhibition of pathogenic microbiota

SUMMARY

The concrete nature of the probiotic effects that the presence of microorganisms (especially lactic acid bacteria: LAB) exercise on larval cultures of fish, it is not well defined, being been able to attribute to different factors or action mechanisms. In fact, the production of diverse antibacterial metabolites (bacteriocins in particular) by many LAB forms are able to constitute the basis of these probiotic effects, as repeatedly described in the literature. Accordingly, the inhibition of fish pathogenic species by extracts of LAB constitutes a rapid method for detecting potential probiotics. By studying the response of four common pathogens of turbot (*Scophthalmus maximus*) to nine potential probiotics, the diversity and mechanisms of effectors in the probiotics were demonstrated to present complex profiles dose-response and non-treatable with conventional models. Proposed modifications allow satisfactory fits and the calculation of useful parameters in the comparison of activities. The results showed that lactic and acetic acid, and not the bacteriocins, are responsible for effects (inhibitory or stimulatory depending on the concentrations considered) in all the cases studied.

Keywords: Lactic acid lactic bacteria, dose-response models, organic acids

INTRODUCTION

One of the most serious problems concerning turbot (*Scophthalmus maximus*) production is high (~85%) and variable larval mortality, generally attributed to the effects of opportunistic microbiota introduced to the system in the live food (Nicolas et al., 1989; Person-Le Ruyet, 1989; Munro et al., 1994). Cultures of phytoplankton, rotifers and crustaceans (*Isochrysis*, *Brachionus*, *Artemia*) used in larval feeding contain high microbial loads (Lesel, 1981; Nicolas et al., 1989). In cultivation tanks and unhatched turbot eggs bacterial genera have been repeatedly detected (*Pseudomonas*, *Aeromonas*, *Vibrio*) which colonize the fish digestive tube in abundance from the moment of bucal opening. The delayed development of the immune system of the animal makes it highly sensitive to such microbiota, whose effects can be aggravated for a deficient feeding. Although antibiotics improve survival (Planas et al., 1994), they also alter the intestinal niche and induce resistant microorganisms, with unpredictable long term effects on human health.

An alternative to antibiotics which has stimulated current interest is based on probiotics, a phenomenon embracing a number of favourable physiological effects for the host and linked to the microbial balance of their intestinal tract (Fuller, 1990). Probiotic is a classical idea in the area of human dietetics (which underlies to the consumption of fermented lactic products) and is extended to animals by promoting the development of fortifying diets for the intestinal microbiota, which improve feeding yields and survival. Probiotic development in aquaculture began upon discovering that bacteria in the intestinal tracts of healthy animals, or bacteria of the same marine medium, exercised antagonistic effects on pathogenic bacteria (Skjermo and Vadstein, 1999; Makridis et al., 2000; Hjelm et al., 2004).

In general, the exact nature of probiotic effects mediated by microorganisms (for example LAB) it has not been perfectly typified and it can be attributed to different and probably interactive factors and mechanisms, in particular noting:

1: Competition between probiotics and opportunistic or pathogenic microorganisms for adherence sites on the mucous, limiting nutrients or other factors of the intestinal niche (Olsson et al., 1992).

2: Inhibition or antibiosis of the unwanted microbiota by the bacteriocins or other metabolites (lactic and acetic acids, diacetyl, butandiol, acetaldehyde, H_2O_2) typical of the lactic bacteria (Gilliland, 1990; Gatesoupe, 1991; Vandenbergh, 1993). The frequent conservation of probiotic activity in cell-free media in corresponding cultures is a clear indication in favour of this effect.

3: Inmunostimulation induced by the probiotics or associated metabolites (Perdigón et al., 1990; Brassart and Schiffrin, 1997; Villamil et al., 2003a; Nikoskelainen et al., 2003; Villamil et al., 2003b; Gullian et al., 2004).

4: Derived colateral benefits of probiotics effects on the alive diets. It has been demonstrated that some lactic acid bacteria prevent the infection of rotifers by several species of *Aeromonas* (Gatesoupe, 1991) and *Vibrio* (Gatesoupe, 1994), increase their growth rate and improve their nutritional quality (Shiri Harzevili et al., 1998; Planas et al., 2004).

In recent years, there has been great interest in the use of LAB and their metabolic products as potential probiotics in aquaculture (Ringø and Gatesoupe, 1998; Gatesoupe, 1999), to improve population growth of rotifer cultures (Shiri Harzevili et al., 1998; Planas et al., 2003; Planas et al., 2004), their nutritional value for turbot larvae (Gatesoupe, 1991) and thus larval survival (García de la Banda et al., 1992). They have also been used in the treatment of disinfectant of *Artemia* nauplii (Gatesoupe, 2002), as growth promoters of *Oreochromis niloticus* (Maurilio

Lara-Flores et al., 2003) and in the increase of the immnunologic response of turbot (Villamil et al., 2003a; Villamil et al., 2003b) or rainbow trout (Sami Nikoskelainen et al., 2003).

Under these conditions, the most rapid and experimentally most simple step for the test of potential probiotic effects consists of determining the inhibitory activity of the cell-free media of different probiotics on pathogenic bacteria associated with larval cultures. In spite of the schematic approach, the study of such activities in several characteristic cases has demonstrated a series of complex profiles (Cabo et al., 1999; Murado et al., 2002; Planas et al., 2003), not describable with conventional dose-response models (Murado et al., 2002).

In this work, diverse postincubated of LAB fermentations (bacteriocins, lactic and acetic acids) were used in dose-response bioassays against to pathogen microorganisms of marine cultures (*Vibrios*). The obtained results demonstrated the inhibition that the lactic and acetic acids cause in the growth of the *Vibrios* (depending on the concentrations considered), as well as the total absence of response of the bacteriocins on these gram (–) bacteria. In the quantification of the experimental results, dose-response models modified from previously described in Murado et al. (2002) were formulated.

MATERIALS AND METHODS

Microbiological methods

Table 1 shows the species and the origin of the bacteria used, comprising nine potential probiotics and four pathogens. Of the pathogens, *Carnobacterium piscicola* is a common indicator in the bioassay of bacteriocins (Vázquez et al., 2003; Vázquez et al., 2004). Pathogen

HQ 221, although used in aquaculture as a probiotic against pathogens such as *Vibrio anguillarum* and *Aeromonas salmonicida* (Austin et al., 1995), was tested here as a pathogen following suggestions by the Larval Physiology Group at the Instituto de Investigacións Mariñas (Vigo), who detected HQ 221 as the major microorganism in cultures with appreciable mortalities from the company *Stolt Sea Farm* (Villamil et al., 2003c). HQ 222, also associated with total larval mortalities (Pérez Lomba, 2001; Villamil et al., 2003d), is presumably a strain of high virulence. Finally, HQ 223 was supplied by the University of Glasgow, where it has been detected, again associated with high mortalities.

Stock cultures of probiotics were stored at –50ºC in powdered skimmed milk suspension with 25% glycerol (Cabo et al., 1999). Marine species were stored under the same conditions, substituting powdered skimmed milk for marine broth (DIFCO). Assay cultures were grown on MRS medium (DIFCO) at 30ºC with orbital shaking at 200 rpm (or marine medium, 22ºC, 100 rpm for marine species). In both cases, inocula (1% v/v) consisted of cellular suspensions from 12-24 hour-aged cultures on the same media and conditions (Planas et al., 2004), adjusted to an OD (700 nm) of 0.900.

Bioassays were carried out in quintruplicate, following methods and equations described in detail previously (Cabo et al., 1999; Murado et al., 2002), with slight modifications for the marine species:

Effector solutions: The complete post-incubation (medium and cells) of each probiotic was buffered with biphthalate-HCl 0.05M; pH=3.5, treated for 3 minutes at 80ºC and centrifuged, discarding the sediment and taking the supernatant as an effector solution. From this supernatant, which can be conserved frozen at –18ºC until required, a series of dilutions were prepared and applied to the suspensions (indicators) of the microorganisms whose response was studied. The

response of *C. piscicola* CECT 4020 (Spanish Type Culture Collection) was taken as a base for standardizing the activities of all effector solutions. Besides the crude extracts, the corresponding dialyzed samples (>1,000 Da) were also tested for eliminating possible interferences of low molecular mass.

Indicator suspensions: These were prepared by centrifugation of the corresponding cultures and resuspension of the sediment in fresh medium at an OD (700 nm) of 0.200. In the case of *C. piscicola*, the medium (MRS) was buffered with biphthalate-NaOH 0.05M; pH=6.0. In the remaining species, marine medium clarified by centrifugation and without buffer was employed (pH below ~7.2 in this medium induces turbidity). These differences do not affect intercomparison (see Results).

Procedure: Prepared with each effector solution a serie of dilutions (in distilled water in order to obtain a suitable interval of concentrations of the active principle), a volume from each of the dilutions was combined with an identical volume of indicator suspension, and incubated for 6 hours at 30ºC (*C. piscicola*) or 22ºC (*Vibrios*) and the OD (700 nm) was measured. Where A_s and A_0 are the OD of the sample and the control, respectively, the proportions of inhibition used to obtain the dose-response models were $I=1-(A_s/A_0)$.

Definition of a ID_{50} and its concentration in the sample: one unit of ID_{50} is defined as the amount of active principle (bacteriocins or organic acids) present, per unit of volume, in a sample that will produce an inhibition of I=0.5 under the specified experimental conditions. The following procedure will give the calculation or the concentration of active principle present in a given sample. A series of dilutions of the sample to be studied is tested according to the procedure described, with the dilution corresponding to I=0.5 being calculated by mathematic

interpolation (see Appendix). Since, by definition, this dilution contains 1 ID_{50}/ml, the concentration in the sample will be the inverse of the corresponding dilution factor.

Analytical methods

At pre-established times, each probiotic culture sample was divided into two aliquots. The first was centrifuged at 5,000 rpm for 15 min, and the sediment washed twice and resuspended in distilled water to an appropriate dilution for measuring the OD at 700 nm. The dry weight was then estimated from a previous calibration curve. The corresponding supernatant was used for the determination of reducing sugars (Bernfeld, 1951), proteins (Lowry et al., 1951), and organic acids by HPLC analysis (refractive-index detector), using an ION-300 (Interaction Chromatography) column, at 65ºC, with 6mM sulphuric acid as a mobile phase (flow=0.4 ml min^{-1}). The second aliquot (previously dialyzed or not, see dialysis methods) was used for the bioassays as described in the previous section.

Dialysis methods

For the bacteriocins retention and the elimination of molecules of low molecular mass were carried out dialysis processes. Samples of 20 ml of postincubated extracts LAB (effector solutions) and membranes of cellulose: the dialysis tubing (Sigma-Aldrich) with threshold size of 1,000 Da were used. These dialysis tubing was placed in laboratory glasses with 3000 ml of water distilled in a room at 4ºC and with soft agitation (magnetic agitator). At pre-established times, we took samples to analyze the levels of reducing sugars and organic acids like as described in the previous section.

Numerical methods

Fitting procedures and parametric estimations calculated from the experimental results were carried out by minimisation of the sum of quadratic differences between observed and model-predicted values, using the non linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel XP spreadsheet.

RESULTS

1. Dialysis kinetics

From a point of view chemical-physics the dialysis is defined as the mass transfer through a semi-permeable membrane of given pore size. Mathematically, dialysis kinetics can be described (excluding the electrokinetic effect of the Donnan balance, *i.e.*, molecular transfer due to the charge gradient) in similar terms to first order kinetics:

$$\frac{dC}{dt} = K(C_e - C)$$

where C is the concentration of solute, with a value of zero (C_e=0) in the exterior of the membrane. Integrating between the limits $C_0 \rightarrow t$=0 and $C_f \rightarrow t$=t one obtains:

$$C_f(t) = C_0 \exp(-Kt) \qquad [1]$$

As an example illustrating the entirety of the applied method, figure 1 shows the results obtained with extracts of Pc 1.02, at 4ºC and a threshold of 1,000 Da, fitted to equation [1] for the principal dialyzable solutes. The kinetic parameters (Acetic acid (60 g mol^{-1} of molecular mass): K=0.438 h^{-1}; Lactic acid (90 g mol^{-1}): K=0.219 h^{-1}; Glucose (180 g mol^{-1}): K=0.086 h^{-1}) demonstrate the logical decrease (large molecules dialyse more slowly than smaller molecules) of the transfer constant K with increasing molecular mass.

2. Effects on pathogenic bacteria

In the figure 2-upper is represented the response of HQ 221 to extracts of Pc 1.02 (dialyzed and not dialyzed) where it can be observed clearly that the dialyzed samples are less active than the crude extracts. Also, the interference in the marine species from the buffer used for the extraction of the samples can be disregarded (figure 2-lower).

In general, all the dose-response curves obtained on bioassays between the pathogenic species and the effectors solutions presented peculiar profiles, with two clearly different routes (figure 3). In the first, a dose increase stimulates growth; in the second the effect is inhibitory with a conventional logistical profile. Moreover, the comparison of the response to crude and dialyzed extracts (which retain the bacteriocins, but not the metabolites of low molecular mass), suggests that the inhibition is more owing to the dialyzable materials rather than the bacteriocins (figure 2). Accordingly, it is reasonable to suppose that stimulation is due to the remainder of dialyzable nutrients (carbohydrates and peptides) present in the medium, and inhibition to the characteristic metabolites of the lactic bacteria. Analogous results (not shown) were obtained in the remainder of the tests.

The results in figure 4 confirm this previous interpretation. In effect, the response to the crude extracts can be reproduced with great accuracy by supplementing the dialyzed samples with lactic and acetic acid or with solutions of pure nisin (Sigma). Finally, the response to solutions of lactic and acetic acid also reproduces the inhibitory effects of the crude extracts, excluding the stimulatory route.

For additional confirmation of the effects of lactic and acetic acid a further series of bioassays was carried out, treating HQ 221 and HQ 222 with a dialyzed extract of Pc 1.02 supplemented with increasing levels of each acid. Figure 5 shows lactic acid against HQ 221, representative of the other situations.

3. A dose-response model for the detected effects

For the mathematical description of the profiles with the two routes, the used resource was of subtracting to a modified logistical equation (Murado et al., 2002), that it describes the route inhibitory, another asymptotic equation capable to describe the stimulatory route, being essentially the decision between von Bertalanffy, Gompertz or other logistical equation an question of goodness fit. Although the treatment appears uncommon in this field, the rationale is the same as for a basic model such as the Heider-London model (potential energy as a function of the bond radius), which make it necessary to add an exponential empirical term (Born-Mayer) for the strength of repulsion to the theoretical parabolic term for the strength of attraction.

The mixed models investigated (the procedures applicable to [4] for the calculation of parameters and other values of interest are described in the appendix) were the following:

1: Modified logistic – Bertalanffy:

$$I = K\left(\frac{1}{1+e^{r(m-D)}} - \frac{1}{1+e^{rm}}\right) - a\left(1-e^{-b\cdot D}\right) \qquad [2]$$

2: Modified logistic – Gompertz:

$$I = K\left(\frac{1}{1+e^{r(m-D)}} - \frac{1}{1+e^{rm}}\right) - ae^{-be^{-c\cdot D}} \qquad [3]$$

3: Logistic – logistic:

$$I = \frac{K}{1+e^{r(m-D)}} - \frac{K'}{1+e^{r'(m'-D)}} \qquad [4]$$

For adequate model selection the following criteria were considered:

1: The physical significance of the parameters, and not only the fit of the corresponding equation (see table of symbolic notations).

2: The robustness of the model, in the sense (Murado et al., 2002) of minimising the effect of experimental error on the parameters of greater practical interest (*e.g.* ID_{50}), which favours the logistical models.

3: The correlation between expected and observed values, which makes model [4] (r=0.9996) slightly preferable to model [3] (r=0.9995).

The response of HQ 222 to dialyzed sampled from Pc 1.02 supplemented with 1 and 2.5 $g.l^{-1}$ of acetic and lactic acid respectively are shown in figure 6 fitted to the three models. In figure 7 the models [3] and [4] are included with the confidence intervals of the means (α=0.05; υ=4). In

both cases satisfactory fits can be appreciated, and also for the remainder of the bioassays (not plotted) treated similarly.

It is clear that if the ID_{50} is calculated using the concentration of the Pc 1.02 extract as an independent variable (dose), the values differ significantly from those obtained using the lactic or acetic acid supplement. If the ID_{50} is calculated (Table 2) considering the total content of acid, the differences lack statistical significance and can be interpreted as estimations of the mean. This demonstrates that the relevant variable is the acid concentration. Table 3 summaries the tested casuistical, along with the values obtained for ID_{50} and the calculation methods applied to the specific case of HQ 223.

4. Joint effect of lactic and acetic acids

The joint effect was studied in HQ 221 and HQ 222, using a dialyzed extract of Pc 1.02 as a basic effector solution and an increasing series of concentration of an acid, each sample was supplemented with an increasing series of the other acid. The concentration increments were 0.5-5 and 1-10 $g.l^{-1}$ for the acetic and lactic acids, respectively.

From equation [4], and applying the criteria described previously (Cabo et al., 2000; Murado et al., 2002; Planas et al., 2003) for modeling potentiator and antagonist effects, the experimental results (for HQ 222) to show in figure 8 suggests a description in terms of equation [5], in which possible auto-inhibitory effects on the parameters *r*, *m* and *K* of each effector are eliminated, and which do not seem pertinent to these results. Table 4 resumes the systematic interpretation of the parametric values.

$$I_{AL} = \frac{K_A}{(1+\beta_L L)}\left[\frac{1}{1+\exp\left(\frac{r_A}{1+\gamma_L L}\left(\frac{m_A}{1+\rho_L L}-A\right)\right)}\right]-$$

$$-\frac{K'_A}{(1+\mu_L L)}\left[\frac{1}{1+\exp\left(\frac{r'_A}{1+\chi_L L}\left(\frac{m'_A}{1+\delta_L L}-A\right)\right)}\right]+$$

$$+\frac{K_L}{(1+\alpha_A A)}\left[\frac{1}{1+\exp\left(\frac{r_L}{1+\gamma_A A}\left(\frac{m_L}{1+\rho_A A}-L\right)\right)}\right]-$$

$$-\frac{K'_L}{(1+\varepsilon_A A)}\left[\frac{1}{1+\exp\left(\frac{r'_L}{1+\chi_A A}\left(\frac{m'_L}{1+\delta_A A}-L\right)\right)}\right] \qquad [5]$$

Although a model with 24 parameters appears “excessive” and requires an elevated number of experimental values (in this case 18×18=324, an amply sufficient number), the model does not increase the probability of type II error. This is due to the fact that it only involves two independent variables (lactic and acetic acid) without risk their irrelevance. Figure 9 shows the response surface for HQ 222, along with the corresponding experimental points and the correlation between expected and observed values (r^2=0.957). The results were very similar for HQ 221 (not represented), with a corresponding correlation coefficient of r^2=0.949.

DISCUSSION

The high mortality rates that take place in the larvae phases of cultures of marine fish as the turbot (*Scophthalmus maximus*) cause big economic lost in the aquaculture world. These

mortalities are frequently associated to bacteria from the *Vibrio* genus (Toranzo al., 1993) as HQ 221 (Villamil et al., 2003c), *V. anguillarum* (Shiri Harzevili et al., 1998) and HQ 222 (Villamil et al., 2003d). Among the possible alternatives for the improvement of this problem, avoiding to the antibiotics, the use of bacteria like the LAB (potential probiotics) has raised great interest during the last decade (Ringø and Gatesoupe, 1998; Gatesoupe, 1999).

Even this way, the concrete mechanisms of action of the probiotic activity, as well as it happens with the probiotic effects in human, are of very diverse nature and they continue being difficult to attribute effects to independent mechanisms (in many cases the probiotic effect is due to the lineal combination of several action mechanisms). Among the mechanisms it can make an appointment: to the inhibition for compounds (organic acids, bacteriocins), the competition for adhesion sites to mucous or the competition phenomena for restrictive nutrients.

Previous results obtained in the increase of the rotifer growth (*Brachionus plicatilis*) mediated for LAB (Planas et to al., 2004) or for organic acids characteristic of their fermentative metabolism (Planas et to al., 2003), as well as the decrease of HQ 221 in *Artemia* culture (Villamil et to al., 2003c), evidence the previously pointed interest.

Our study proposes in a clear way and from a quantitative perspective, the inhibitory mechanism that the lactic and acetic acids, and not the bacteriocins, causes in the growth of several *Vibrios*. The activity lack that the bacteriocins present is due to the impossibility that these peptides have of acceding to the plasmatic membrane of bacteria gram (–) and can to act in the formation of transmembrane pores. On the contrary, the lactic and acetic acids in their undissociated form (generalmente metabolitos primarios de las fermentaciones lácticas de las LAB) possess the ability to cross membranas of microorganisms, become dissociated incide and acidify the

interior promoting the expulsión of H+ ions from the cells and causing uncouple of the Na^{+}-K^{+} (ATPase) pump (Gonçalves et al., 1997).

The dose-response curves obtained of the corresponding bioassays present complex experimental profiles where two clearly different sections or routes can be appreciated. In the first, the increase of the acid dose stimulates the growth of the *Vibrios* (section that doesn't appear when the samples tested were dialyzed previously), and another in which the increase of the acid dose inhibits the pathogen bacteria with a typically logistical profile. It is evident that the stimulative effect is due to the remains of the fermentative metabolism of the samples of LAB (proteins and sugars) and the inhibitor effect (starting from certain concentration of acids) to the lactic or acetic acids.

Using purified dose-response models from a previous work (Murado et al., 2002), diverse mechanistically reasonable modifications for such descriptions are proposed in this work. The modified models allow satisfactory fits, the calculation of useful parameters in the comparison of activities and demonstrate that lactic and acetic acid are responsible for the effects in all the cases studied.

Symbolic notations used		
EQUATIONS	PARAMETERS	
Dialysis kinetics	C_f:	concentration in the interior of the membrane at time *t*. Dimensions: $g.l^{-1}$.
	C_0:	initial concentration in the interior of the membrane. Dimensions ($g\ l^{-1}$).
	K:	Transfer constant of the solute across the membrane. Dimensions (t^{-1}).
Modified logistic + Bertalanffy	*I*,*D*:	proportion of inhibition and dose, respectively.
	K:	empirically determined parameter. Dimensions *I*.
	r:	specific inhibition coefficient. Dimensions D^{-1}.
	m:	empirically determined parameter. Dimensions *D*.
	a:	empirically determined parameter. Dimensions *I*.
	b:	empirically determined parameter. Dimensions D^{-1}.
Modified logistic + Gompertz	*I*,*D*:	proportion of inhibition and dose, respectively.
	K:	empirically determined parameter. Dimensions *I*.
	r:	specific inhibition coefficient. Dimensions D^{-1}.
	m:	empirically determined parameter. Dimensions *D*.
	a:	empirically determined parameter. Dimensions *I*.
	b:	empirically determined parameter. Dimensionless.
	c:	empirically determined parameter. Dimensions D^{-1}.
Logistic + logistic	*I*, *D*:	proportion of inhibition and dose, respectively.
	K, *K'*:	empirically determined parameter. Dimensions *I*.
	r, *r´*:	specific inhibition coefficients. Dimensions D^{-1}.
	m, *m'*:	empirically determined parameter. Dimensions *D*.

APPENDIX

'Logistic + logistic' equation:

$$I = \frac{K}{1+e^{r(m-D)}} - \frac{K'}{1+e^{r'(m'-D)}} \qquad [4]$$

Determination of minimum (I_0) and maximum (I_∞) inhibition:

$$I_0 = \lim_{t\to 0} \frac{K}{1+e^{rm}} - \frac{K'}{1+e^{r'm'}} \quad , \ \forall r, m, r', m' \qquad [4.1]$$

$$I_{máx} = I_\infty = \lim_{t\to \infty} \frac{K}{1+e^{r(m-\infty)}} - \frac{K'}{1+e^{r'(m'-\infty)}} =$$
$$= \frac{K}{1+e^{-\infty}} - \frac{K'}{1+e^{-\infty}} = K - K' \quad ; \quad \forall r, m, r', m' > 0 \qquad [4.2]$$

ID_{50} search: The value of inhibition is, by definition:

$$\frac{I_{máx}}{2} = \frac{K-K'}{2} = \frac{K}{1+e^{r(m-D)}} - \frac{K'}{1+e^{r'(m'-D)}} \qquad [4.3]$$

and the corresponding dose is:

$$ID_{50} = m - \frac{1}{r}\ln\left(\frac{K-K'+(K+K')e^{r'(m'-D_{i-1})}}{K+K'+(K-K')e^{r'(m'-D_{i-1})}}\right) \qquad [4.4]$$

transcendent equation which can be resolved by numerical iteration until $D_i=D_{i-1}$.

In absence of a descending route (*i.e.*, when $K'=r'=m'=0$), the equation is reduced to a logistical equation (Murado et al., 2002), where:

$$ID_{50} = m - \frac{1}{r}\ln\left(\frac{K+K}{K+K}\right) = m - \frac{1}{r}\ln 1 = m \qquad [4.5]$$

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Hazel Duncan (University of Glasgow, Scotland, UK), Dr. Ana Riaza (SSF, Norway) and Dr. Miguel Planas (IIM-CSIC, Spain) for the provision of the microbial strains. This work was supported by the project *Improved procedures for flatfish larval rearing through the use of probiotic bacteria* (PROBE) from European Commission (contract no. *Q5RS-2000-31457)*. José Antonio Vázquez Álvarez was a doctoral fellow of the Deputación de Pontevedra at the Instituto de Investigacións Mariñas de Vigo (CSIC). We also wanted to thank to the referees for their comments in the improvement of the work.

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TABLES

TABLE 1: Bacteria used

Species	Reference	Key IIM: origin
Lactobacillus acidophilus	CECT 903	Lb 1.02: acidophilus milk
Lactobacillus brevis	CECT 216	Lb 2.01: beer
Lactobacillus casei ssp. casei	CECT 4043	Lb 3.04: Majorero cheese
Lactobacillus delbrueckii ssp. lactis	CECT 282	Lb 4.01: fermented meat
Lactobacillus helveticus	CECT 541	Lb 6.04: Gruyere cheese
Lactobacillus plantarum	CECT 221	Lb 8.02: grass silage
Lactococcus lactis ssp. lactis	CECT 539	Lc 1.04: milk
Leuconostoc mesenteroides ssp. mesenteroides	CECT 4046	Ln 3.07: pressed curt
Pediococcus acidilactici	NRRL B-5627	Pc 1.02: cured meat
Carnobacterium piscicola	CECT 4020	Cb 1.01: cultivated trout
Vibrio alginolyticus	SSF 1	HQ 221: cultivated turbot
Vibrio pelagius	SSF 2	HQ 222: cultivated turbot
Vibrio splendidus	DMC-1	HQ 223: cultivated turbot

CECT: Spanish Type Culture Collection.
NRRL: Northern Regional Research Laboratory (Peoria, Illinois, USA).
DMC: University of Glasgow
SSF: Stolt Sea Farm
Key IIM: Abbreviate notation used in this work

TABLE 2: ID_{50} and standard deviation to increasing concentration of lactic and acetic acid

SUPPLEMENT	SPECIES	mean concentration of acid to ID_{50} (g l^{-1})	standard deviation (g l^{-1})
Lactic	HQ 221	0.3216	0.0529
Acetic	"	0.1925	0.0274
Lactic	HQ 222	0.3920	0.0819
Acetic	"	0.2344	0.0167

TABLE 3: ID_{50} obtained in the bioassays that suggest. Fitted with the modified logistic–Gompertz model [3], Logistic – logistic [4] and modified logistic (Cb 1.01) (Murado et al., 2002).

EXTRACT	HQ 223 [3]	HQ 223 [4]	Cb 1.01 (modified logistic).
Pc 1.02 without dialyze	24.33 / 26.59	29.57 / 28.65	264.36
Lb 6.04 without dialyze	34.15	34.00	9.89
Lb 6.04 dialyzed	2.15	2.15	1.59
Lb 2.01 without dialyze	21.34	20.65	7.17
Lb 2.01 dialyzed	without inhibition	without inhibition	1.75
Lb 3.04 without dialyzed	30.00	30.28	9.79
Lb 3.04 dialyzed	-	-	1.22
Ln 3.07 without dialyzed	34.96	34.91	10.57
Ln 3.07 dialyzed	2.10	2.10	1.25
Lc 1.04 without dialyzed	17.17	17.16	10.31
Lc 1.04 dialyzed	without inhibition	without inhibition	0.32
Lactic 10 g l^{-1}	25.83	23.15	-
Acetic 5 g l^{-1}	26.51	18.25	-
Lactic 10 g l^{-1}-Acetic 5 g l^{-1}	42.17	42.14	-
Lactic 8 g l^{-1}-Acetic 4 g l^{-1}	-	-	4.45
Lb 1.02 without dialyzed	22.64	23.04	6.11
Lb 4.01 without dialyzed	17.37	17.37	5.05
Lb 8.02 without dialyzed	37.67	26.54	7.59
Nisin 3 g l^{-1}	without inhibition	without inhibition	84.23

TABLE 4: Parameters of the model [5] to description the joint effects of the lactic and acetic acids on HQ 222.

acetic effects		lactic effects		lactic effects on acetic		acetic effects on lactic	
K_A= 0.554	K'_A= 0.112	K_L= 3.200	K'_L= 2.402	β_L= 3.887	μ_L= 0.866	α_A= 0.008	ε_A= 0.011
r_A= 33.034	r'_A= 48.714	r_L= 11.130	r'_L= 8.630	γ_L= 0.203	χ_L= 0.487	γ_A= 0.571	χ_A= 0.395
m_A= 0.604	m'_A= 12.344	m_L= 0.497	m'_L= 0.605	ρ_L= 22.675	δ_L= 115.77	ρ_A= 14.850	δ_A= 35.452

FIGURE CAPTIONS

Figure 1: Dialysis kinetics of the post incubated medium from Pc 1.02 on MRS. Experimental data (points) and fitted (line) to the equation [1]. : glucose; ○: lactic acid; ◇: acetic acid.

Figure 2: Response of the HQ 221 to Pc 1.02 crude extracts and dialyzed (up), as well as of buffer biphthalate-HCl; pH=3.5 used in substitution of the extracts (down). Besides of the dose scale of agreement with the conventional approach used for the bacteriocins extracts (D) (Murado et al., 2002), in the abscissa are given the lactic (L), acetic (A) and hydrochloric acids concentrations (HCl) in g l^{-1} (L and A) or mM (HCl) on the different dilutions. Experimental results fitted to the equation [4].

Figure 3: Response of pathogen HQ 221 to dialyzed of Pc 1.02 supplemented with acetic (A) and lactic acid (B). Representation of the routes 1 and 2 (see text). Experimental results fitted to the equation [4].

Figure 4: Response of the pathogen HQ 221 to watery solutions of acetic (A) and lactic acid (B), as well as pure nisin (C) and nisin supplemented with 20 g l^{-1} of lactic acid (D). Experimental results fitted to the equation [4].

Figure 5: Dose-response relationships of HQ 221 treated with dialyzed extracts of Pc 1.02 supplemented with different levels of lactic acid. A, B, C, D, E, F: 10; 7.5; 5; 2.5; 1 and 0.5 g l^{-1} of lactic acid, respectively. Data not fitted.

Figure 6: Fit to the models [2], [3], [4] (above A, centre B, below C) of the response of HQ 222 to increasing dose of Pc 1.02 dialyzed extracts supplemented with 2.5 g l^{-1} of lactic acid and 1 g l^{-1} of acetic acid. To the right, the corresponding correlations between expected and observed values.

Figure 7: The same data of the figure 5, contained the confidence intervals (α=0.05 ν=4) in the cases of fits to the equations [3] and [4] (A and B, respectively).

Figure 8: Joint effect of lactic and acetic acids on HQ 222, according to the model [5]. Abscissas: acetic acid; A to J: 0.004; 0.016; 0.031; 0.063; 0.125; 0.234; 0.313; 0.469; 1.25 and 5 g l^{-1} of lactic acid.

Figure 9: Joint effect of lactic and acetic acids on HQ 222, according to the model [5]. It is an alternative representation (3D) of the data shown in the figure 8.

FIGURE 1

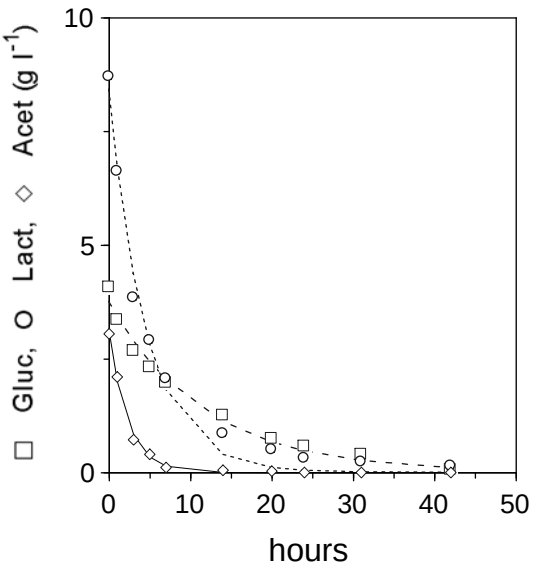


FIGURE 2

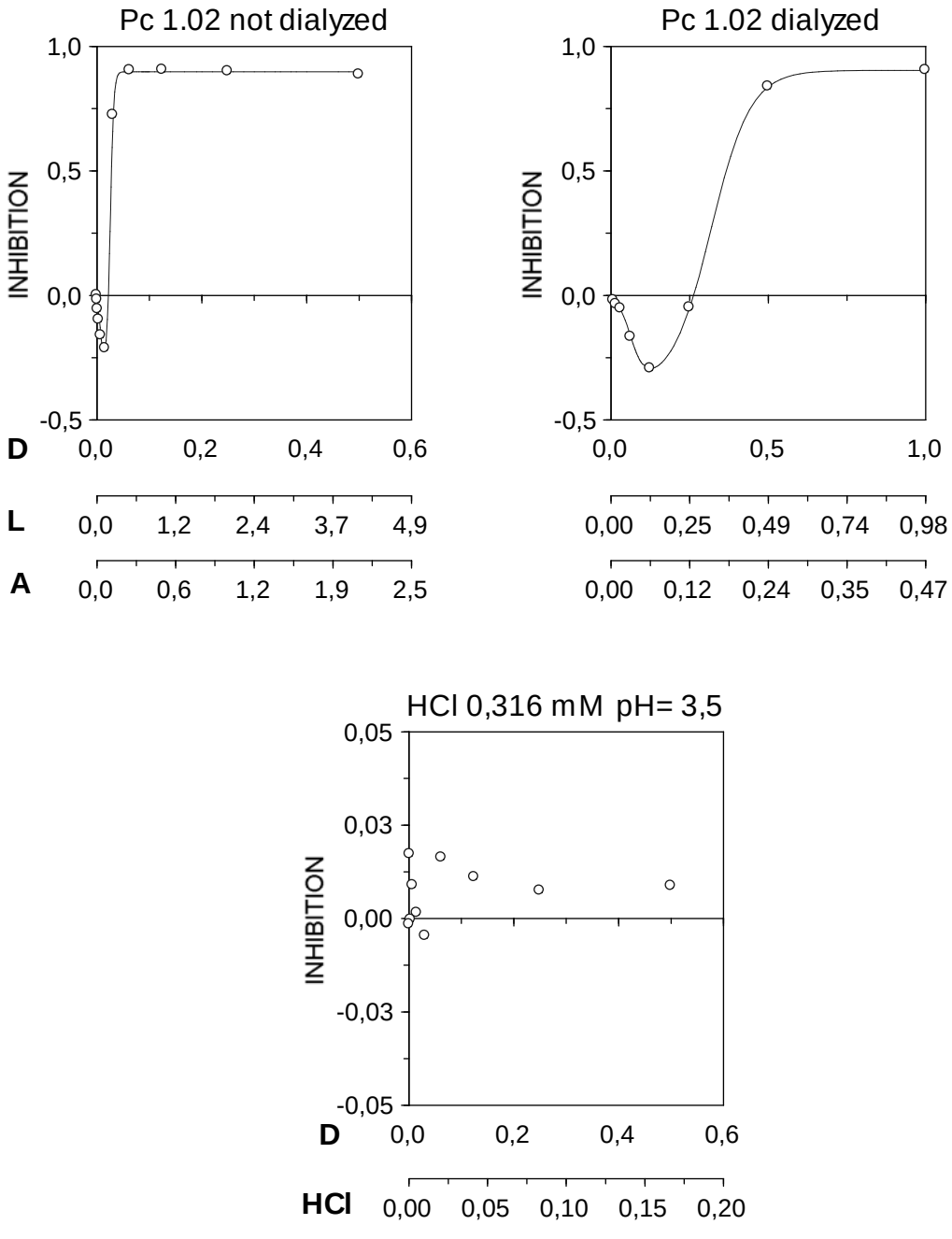

Pc 1.02 not dialyzed
Pc 1.02 dialyzed
INHIBITION
1,0
0,5
0,0
-0,5
D
0,0 0,2 0,4 0,6
L
0,0 1,2 2,4 3,7 4,9
A
0,0 0,6 1,2 1,9 2,5
0,0 0,5 1,0
0,00 0,25 0,49 0,74 0,98
0,00 0,12 0,24 0,35 0,47
HCl 0,316 mM pH= 3,5
0,05
0,03
0,00
-0,03
-0,05
HCl
0,00 0,05 0,10 0,15 0,20

FIGURE 3

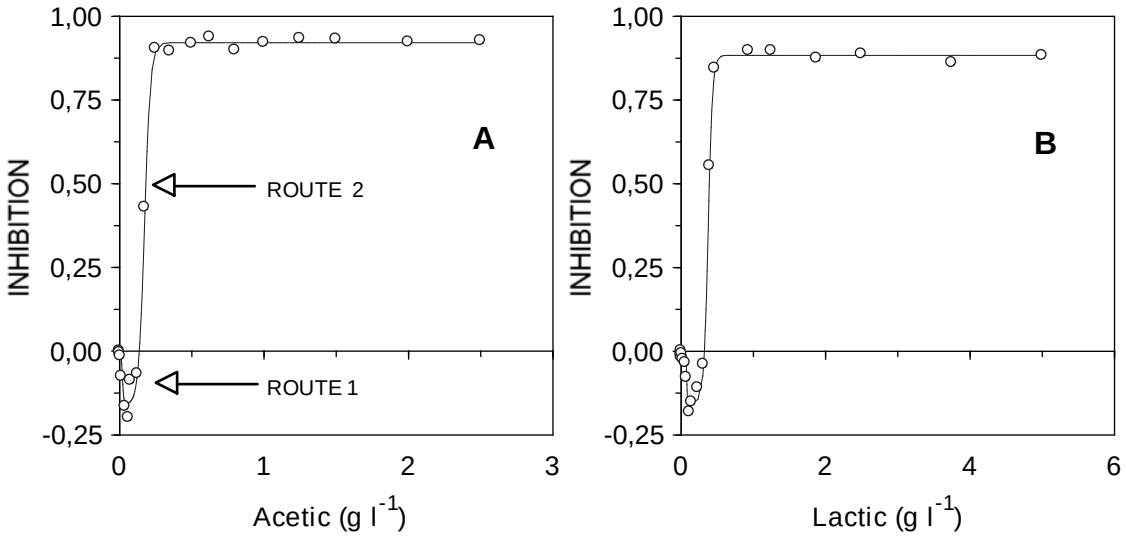

1,00
0,75
0,50
0,25
0,00
-0,25
INHIBITION
A
ROUTE 2
ROUTE 1
0
1
2
3
Acetic (g l-1)
B
4
6
Lactic (g l-1)

FIGURE 4

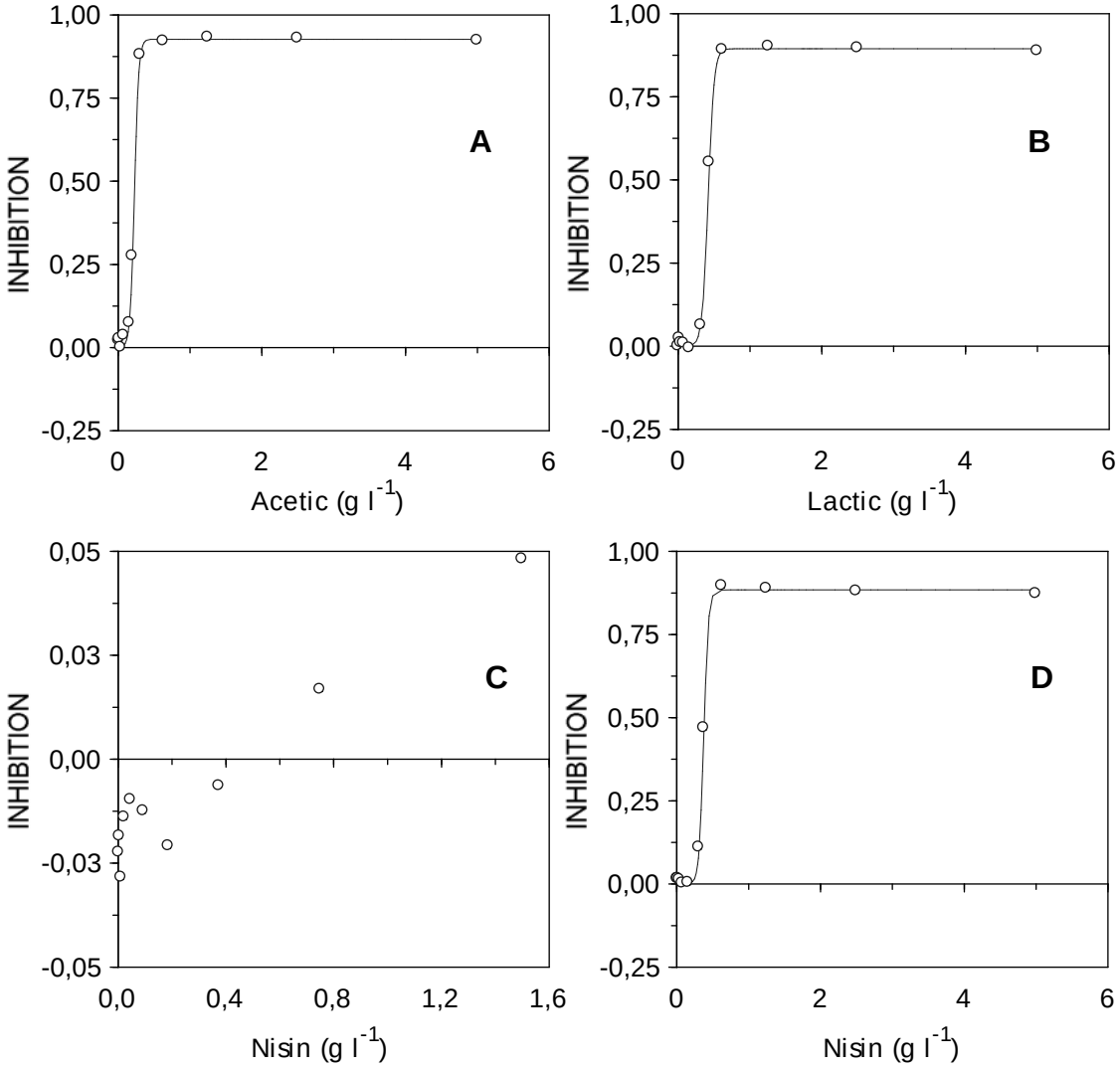

A
B
C
D
INHIBITION
Acetic (g l-1)
Lactic (g l-1)
Nisin (g l-1)
1,00
0,75
0,50
0,25
0,00
-0,25
0,05
0,03
-0,03
-0,05
0
2
4
6
0,0
0,4
0,8
1,2
1,6

FIGURE 5

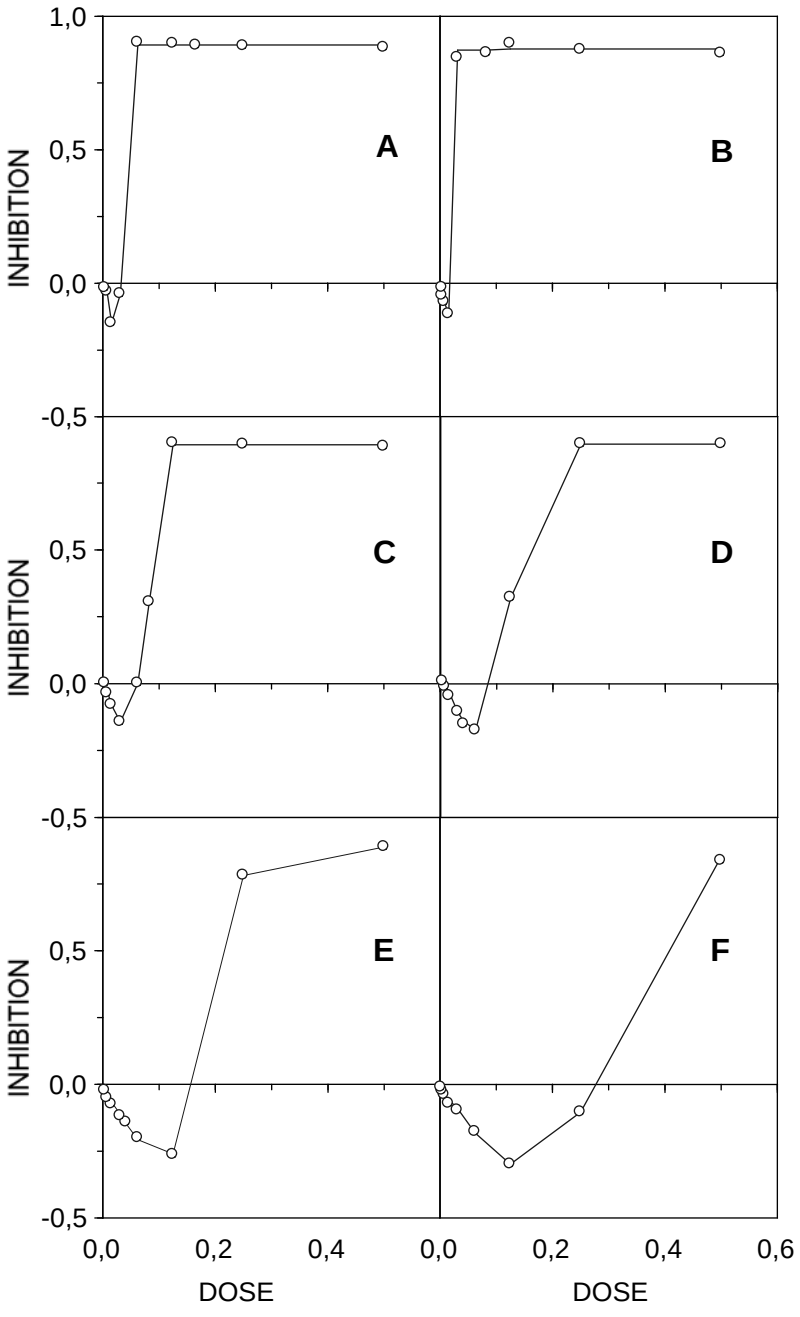


FIGURE 6

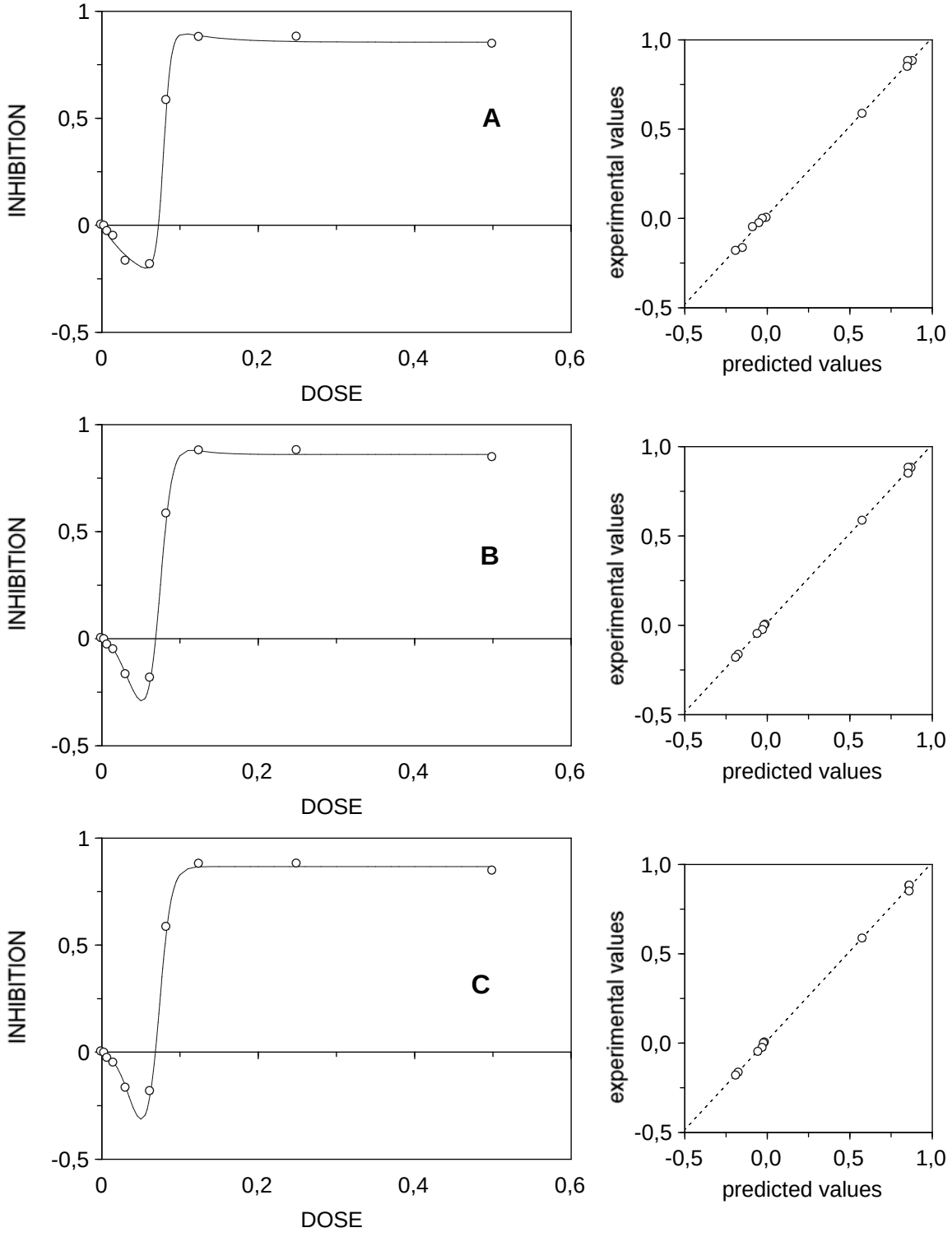

INHIBITION
DOSE
A
B
C
experimental values
predicted values
1
0,5
0
-0,5
0
0,2
0,4
0,6
1,0
0,5
0,0
-0,5
-0,5
0,0
0,5
1,0

FIGURE 7

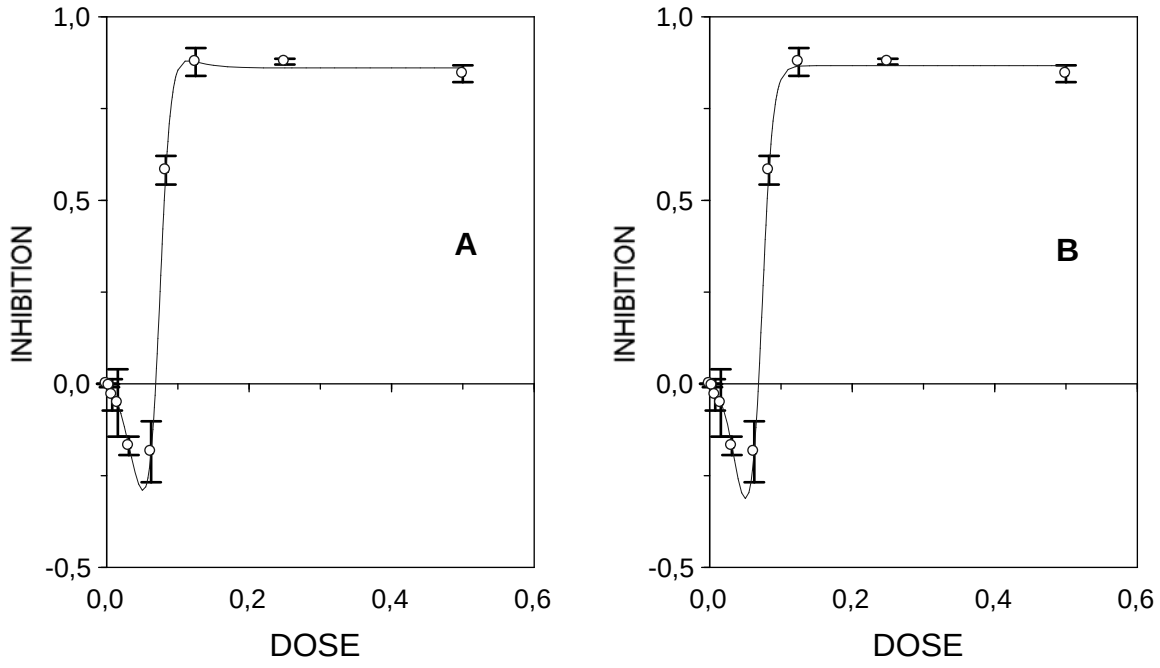

1,0
0,5
0,0
-0,5
INHIBITION
A
0,0
0,2
0,4
0,6
DOSE
1,0
0,5
0,0
-0,5
INHIBITION
B
0,0
0,2
0,4
0,6
DOSE

FIGURE 8

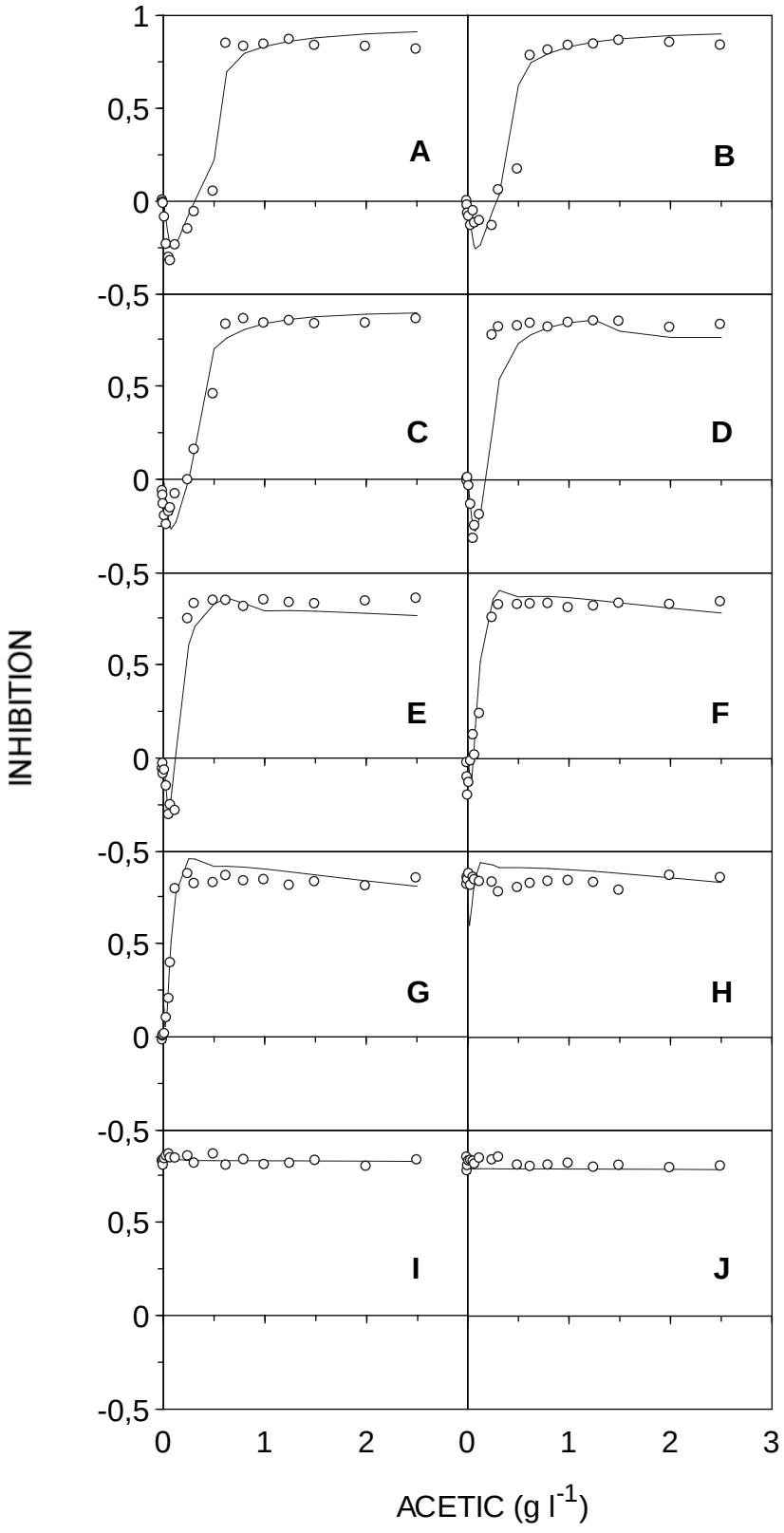
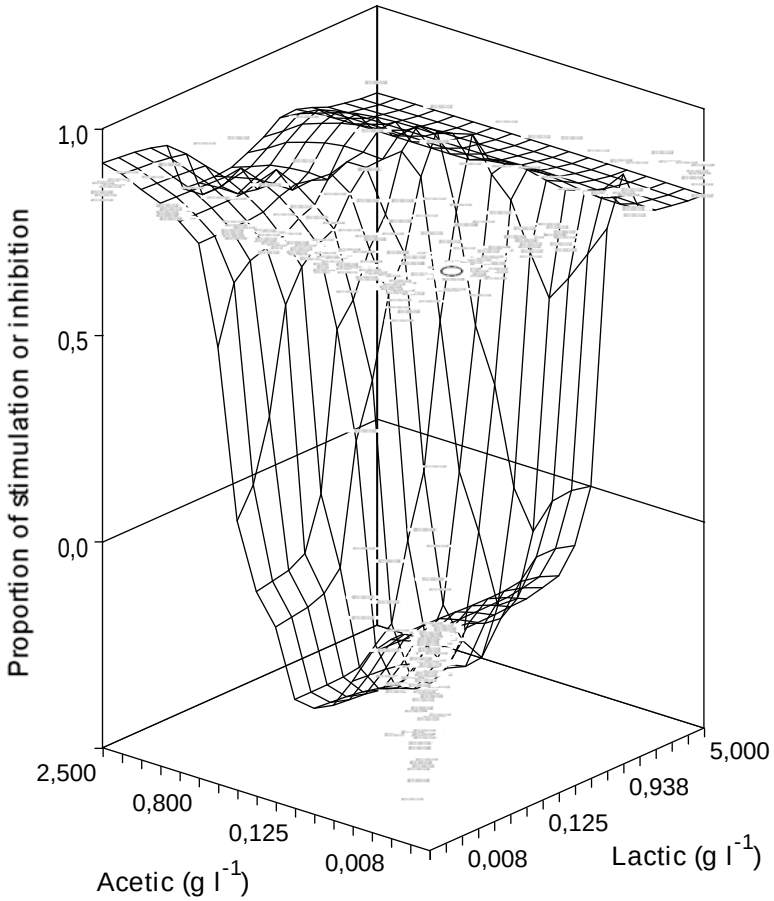
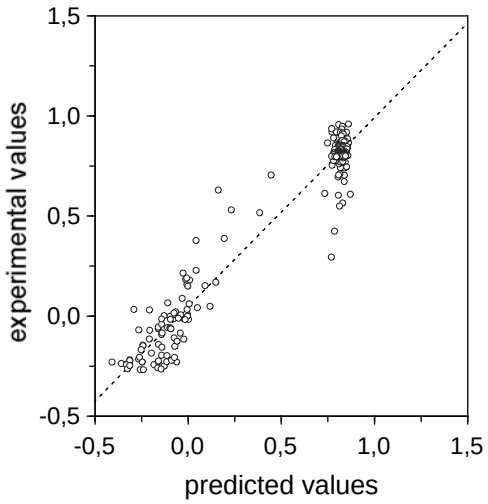


FIGURE 9


Proportion of stimulation or inhibition
1,0
0,5
0,0
2,500
0,800
0,125
0,008
Acetic (g l-1)
0,008
0,125
0,938
5,000
Lactic (g l-1)


experimental values
1,5
1,0
0,5
0,0
-0,5
-0,5
0,0
0,5
1,0
1,5
predicted values